Amendments to the Specification

Please amend the published specification, US 2002/0119489 A1, published Aug. 29, 2002, as follows (changes shown in tabular form first, then in paragraph form):

Location	Currently reads	Should read
¶ 16	quantitiation	quantitation
¶ 24	buyrylcholinesterase	butyrylcholinesterase
¶ 24	Tryptophane	Tryptophan
¶ 27	butrylcholinesterase	butyrylcholinesterase
¶ 28	Denhart's	Denhardt's
¶ 28	Denhart's	Denhardt's
¶ 28	Ansubel	Ausubel
¶ 28	complimentary	complementary
¶ 28	detectible	detectable
¶ 30	complimentary	complementary
¶ 36	an another	another
¶ 36	Tryptophane	Tryptophan
¶ 47	Tryptophane	Tryptophan
¶ 51	^{65}Cys-^{92}Cys, ^{252}Cys-^{263}Cys, and ^{400}Cys-^{519}Cys	^{65}Cys-^{92}Cys, ^{252}Cys-^{263}Cys, and ^{400}Cys-^{519}Cys
¶ 52	butryrylcholinesterase	butyrylcholinesterase
¶ 52	butryrylcholinesterase	butyrylcholinesterase
¶ 56	^{65}Cys-^{92}Cys, ^{252}Cys-^{263}Cys, and ^{400}Cys-^{519}Cys	^{65}Cys-^{92}Cys, ^{252}Cys-^{263}Cys, and ^{400}Cys-^{519}Cys
¶ 63	butyrylchoinesterase	butyrylcholinesterase
¶ 65	Table 1.	Table 2.
¶ 67	Biotechnoloqy	Biotechnology
¶ 70	occuring	occurring
¶ 85	Ansubel	Ausubel
¶ 85	for example,by	for example, by
¶ 87	established	establish
¶ 93	descibed	described
¶ 96	polypetides	polypeptides
¶ 96	individual	individuals
¶ 96	*Neurosura.*	*Neurosurg.*
¶ 104	Tryptophane	Tryptophan
¶ 107	Tryptophane	Tryptophan
¶ 111	synthesase	synthetase
¶ 115	Piscatawy	Piscataway
¶ 115	butyrylcholinesterasae	butyrylcholinesterase

Location	Currently reads	Should read
¶ 115	variants was	variants were
¶ 116	variants	variant
¶ 123	phenyloxazolyl0-benzene	phenyloxazolyl)-benzene
¶ 123	palced	placed
¶ 137	^{65}Cys-^{92}Cys, ^{252}Cys-^{263}Cys, and ^{400}Cys-^{519}Cys	^{65}Cys-^{92}Cys, ^{252}Cys-^{263}Cys, and ^{400}Cys-^{519}Cys
¶ 138	phosphoramadites	phosphoramidites
¶ 139	are be	are
¶ 139	are	is
¶ 140	butyrylcholinest	butyrylcholinesterase.
¶ 141	occurs	occurs
¶ 152	Table 6	[¶]Table 6
¶ 169	is be expected	is expected
¶ 179	*Moderm*	*Modern*

Changes shown above in table form are shown below in paragraph form.

¶ 16:

Figure 5 shows (A) the correlation between the HPLC assay and the isotope tracer assay as demonstrated by plotting the ~~quantitiation~~ **quantitation** of benzoic acid formation by both methods, and (B) the K_m for cocaine hydrolysis activity of horse butyrylcholinesterase using the Lineweaver-Burk double-reciprocal plot.

¶ 24:

A butyrylcholinesterase variant can have a single amino acid alteration as well as multiple amino acid alterations compared to ~~buyrylcholinesterase~~ **butyrylcholinesterase**. A specific example of a butyrylcholinesterase variant is butyrylcholinesterase having the amino acid ~~Tryptophane~~ **Tryptophan** at position 328, of which the amino acid sequence and encoding nucleic acid sequence is shown in Figure 1 and designated as SEQ ID NOS: 2 and 1, respectively. Additional examples of butyrylcholinesterase variants are butyrylcholinesterase having the amino acid Glycine at position 287, of which the amino acid sequence and nucleic acid sequence are described herein and designated SEQ ID NOS: 4 and

3, respectively; butyrylcholinesterase having the amino acid Glutamine at position 285, of which the amino acid sequence and nucleic acid sequence are described herein and designated SEQ ID NOS: 6 and 5, respectively; and butyrylcholinesterase having the amino acid Serine at position 285, of which the amino acid sequence and nucleic acid sequence are described herein and designated SEQ ID NOS: 8 and 7, respectively. The term is also intended to include butyrylcholinesterase variants encompassing, for example, modified forms of naturally occurring amino acids such as D-stereoisomers, non-naturally occurring amino acids, amino acid analogues and mimetics so long as such variants have substantially the same amino acid sequence as butyrylcholinesterase and exhibit cocaine hydrolysis activity. A butyrylcholinesterase variant of the invention can have one or more amino acid alterations outside of the regions determined or predicted to be important for cocaine hydrolysis activity herein. Furthermore, a butyrylcholinesterase variant of the invention can have one or more additional modifications that do not significantly change its cocaine hydrolysis activity. A butyrylcholinesterase variant of the invention can also have increased stability compared to butyrylcholinesterase.

¶ 27:

As used herein, the term "corresponding to" refers to an amino acid sequence that is substantially the same as a reference amino acid sequence. The amino acid sequence can occupy the same or different amino acid positions relative to the reference polypeptide, fragment or segment. It is understood that, while butyrylcholinesterases of different species origin as well as allelic variations will have substantially identical amino acid sequences, the physical locations as well as the size of a particular amino acid sequence may vary. Therefore, the amino acids making up a given segment in a ~~butyrylcholinesterase~~ **butyrylcholinesterase** or butyrylcholinesterase variant may not be in the same physical location or occupy the identical amino acid positions as in the reference butyrylcholinesterase or butyrylcholinesterase variant. For example, butyrylcholinesterases of different species origin as well as allelic variations have substantially similar amino acid sequences, but the amino acid positions making up a region may not correspond to those recited for SEQ ID

NOS: 9 through 15. For example, a region that is substantially similar in amino acid sequence to the region designated as SEQ ID NO: 9 may not occupy amino acid positions 68-82 in a non-human butyrylcholinesterase or an allelic variation of any species origin, but is nevertheless encompassed by the present invention.

¶ 28:

As used herein, the term "substantially the same" in reference to a nucleic acid molecule of the invention or a fragment thereof includes sequences having one or more additions, deletions or substitutions with respect to the reference sequence, so long as the nucleic acid molecule retains its ability to selectively hybridize with the subject nucleic acid molecule under moderately stringent conditions, or highly stringent conditions. The term "moderately stringent conditions," as used herein, refers to hybridization conditions equivalent to hybridization of filter-bound nucleic acid in 50% formamide, 5 X ~~Denhart's~~ **Denhardt's** solution, 5 X SSPE, 0.2% SDS at 42°C, followed by washing in 0.2 X SSPE, 0.2% SDS, at 50°. As used herein, "highly stringent conditions" are conditions equivalent to hybridization of filter-bound nucleic acid in 50% formamide, 5 X ~~Denhart's~~ **Denhardt's** solution, 5 X SSPE, 0.2% SDS at 42°C, followed by washing in 0.2 X SSPE, 0.2% SDS, at 65°. Other suitable moderately stringent and highly stringent hybridization buffers and conditions are well known to those of skill in the art and are described, for example, in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, New York (1992) and in ~~Ansubel~~ **Ausubel** et al., *Current Protocols in Molecular Biology*, John Wiley and Sons, Baltimore, MD (1998). Thus, it is not necessary that two nucleic acids exhibit sequence identity to be substantially ~~complimentary~~ **complementary**, only that they can specifically hybridize or be made to specifically hybridize without ~~detectible~~ **detectable** cross reactivity with other similar sequences.

¶ 30:

As used herein, the term "fragment" when used in reference to a nucleic acid encoding the claimed polypeptides is intended to mean a nucleic acid having substantially the same sequence as a portion of a nucleic acid encoding a polypeptide of the invention or segments thereof. The nucleic acid fragment is sufficient in length and sequence to selectively hybridize to a butyrylcholinesterase variant encoding nucleic acid or a nucleotide sequence that is ~~complimentary~~ **complementary** to a butyrylcholinesterase variant encoding nucleic acid. Therefore, fragment is intended to include primers for sequencing and polymerase chain reaction (PCR) as well as probes for nucleic acid blot or solution hybridization.

¶ 36:

As used herein, the term "alteration" is intended to refer to a modification at an amino acid position of butyrylcholinesterase. An amino acid alteration therefore can be a substitution, deletion or any other structural modification at an amino acid position. An amino acid alteration can occur directly at the amino acid level or result from translation of a nucleic acid encoding an amino acid alteration. An amino acid alteration can lead to the replacement of an amino acid with ~~an another~~ another amino acid or with an amino acid analog. Examples of an amino acid alteration include the amino acid substitution of Alanine (A) with ~~Tryptophane~~ **Tryptophan** (W) resulting in the butyrylcholinesterase variant designated SEQ ID NO: 2; the amino acid substitution of Serine (S) with Glycine (G) resulting in the butyrylcholinesterase variant designated SEQ ID NO: 4; the amino acid substitution of Proline (P) with Glutamine (Q) resulting in the butyrylcholinesterase variant designated SEQ ID NO: 6; and the amino acid substitution of Proline (P) with Serine (S) resulting in the butyrylcholinesterase variant designated SEQ ID NO: 8.

¶ 47:

The invention provides a butyrylcholinesterase variant shown as SEQ ID NO: 2 that has substantially the same amino acid sequence as human butyrylcholinesterase, but includes at amino acid position 328 of human butyrylcholinesterase (SEQ ID NO: 17) a ~~Tryptophane~~ **Tryptophan** (W) substitution in place of the encoded Alanine (A) residue. The A328W butyrylcholinesterase variant (SEQ ID NO: 2) was obtained by PCR site-directed mutagenesis of human butyrylcholinesterase as described in Example I below and exhibits at least a fifteen-fold increase in cocaine hydrolysis activity compared to human butyrylcholinesterase.

¶ 51:

A butyrylcholinesterase variant of the invention can be prepared by a variety of methods well known in the art. If desired, random mutagenesis can be performed to prepare a butyrylcholinesterase variant of the invention. Alternatively, as disclosed herein, site-directed mutagenesis based on the information obtained from structural, biochemical and modeling methods described herein can be performed to target those amino acids predicted to be important for cocaine hydrolysis activity. For example, molecular modeling of cocaine in the active site of butyrylcholinesterase can be utilized to predict amino acid alterations that allow for higher catalytic efficiency based on a better fit between the enzyme and its substrate. As described herein, residues predicted to be important for cocaine hydrolysis activity include 8 hydrophobic gorge residues and the catalytic triad residnes. Furthermore, it is understood that amino acid alterations of residues important for the functional structure of a butyrylcholinesterase variant, which include the cysteine residues ~~^{65}Cys-^{92}Cys, ^{252}Cys-^{263}Cys, and ^{400}Cys-^{519}Cys~~ **^{65}Cys-^{92}Cys, ^{252}Cys-^{263}Cys, and ^{400}Cys-^{519}Cys** involved in intrachain disulfide bonds are generally not altered in the preparation of a butyrylcholinesterase variant that has cocaine hydrolysis activity.

¶ 52:

Following mutagenesis of ~~butyrylcholinesterase~~ **butyrylcholinesterase** or a ~~butyrylcholinesterase~~ **butyrylcholinesterase** variant expression, purification and functional characterization of the butyrylcholinesterase variant can be performed by methods well known in the art. As disclosed below, a butyrylcholinesterase variant can be expressed in an appropriate host cell line and subsequently purified and characterized for cocaine hydrolysis activity. Butyrylcholinesterase variants characterized as having significantly increased cocaine hydrolysis activity can subsequently be used in the methods of hydrolyzing a cocaine-based substrate as well as the methods of treating a cocaine-induced condition described below.

¶ 56:

If desired, random segments of a butyrylcholinesterase variant can be prepared and tested in the assays described herein. A fragment having any desired boundaries and modifications compared to the amino acid sequence of the reference butyrylcholinesterase or butyrylcholinesterase variant of the invention can be prepared. Alternatively, available information obtained by the structural, biochemical and modeling methods described herein can be used to prepare only those fragments of a butyrylcholinesterase variant that are likely to retain the cocaine hydrolysis activity of the parent variant. As described herein, residues predicted to be important for cocaine hydrolysis activity include 8 hydrophobic gorge residues and the catalytic triad residues. Furthermore, residues important for the functional structure of a butyrylcholinesterase variant include the cysteine residues ~~^{65}Cys-^{92}Cys, ^{252}Cys-^{263}Cys, and ^{400}Cys-^{519}Cys~~ **^{65}Cys-^{92}Cys, ^{252}Cys-^{263}Cys, and ^{400}Cys-^{519}Cys** involved in intrachain disulfide bonds. Therefore, a functional fragment can be a truncated form, region or segment of the reference butyrylcholinesterase variant designed to possess most or all of the residues critical for cocaine hydrolysis activity or functional structure so as to retain equivalent cocaine hydrolysis activity. Similarly, a functional fragment can include non-peptidic structural elements that serve to mimic structurally or functionally important

residues of the reference variant. Also included as butyrylcholinesterase variants of the invention are fusion proteins that result from linking a butyrylcholinesterase variant or functional fragment thereof to a heterologous protein, such as a therapeutic protein, as well as fusion constructs of nucleic acids encoding such fusion proteins. Fragments of nucleic acids that can hybridize to a butyrylcholinesterase variant or functional fragment thereof are useful, for example, as hybridization probes and are also encompassed by the claimed invention.

¶ 63:

In addition to structural modeling of butyrylcholinesterase, biochemical data can be used to determine or predict regions of butyrylcholinesterase important for cocaine hydrolysis activity when preparing a focused library of ~~butyrylcholoinesterase~~ **butyrylcholinesterase** variants. In this regard, the characterization of naturally occurring butyrylcholinesterase variants with altered cocaine hydrolysis activity is useful for identifying regions important for the catalytic activity of butyrylcholinesterase. Similarly, site-directed mutagenesis studies can provide data regarding catalytically important amino acid residues as reviewed, for example, in Schwartz et al., *Pharmac. Ther.* 67: 283-322 (1992), which is incorporated by reference.

¶ 65:

Once a number of regions has been identified by any method appropriate for determination of regions important for cocaine hydrolysis, or combination thereof, each region can be randomized across some or all amino acid positions to create a library of variants containing the wild-type amino acid plus one or more of the other nineteen naturally occurring amino acids at one or more positions within each of the regions. Seven regions of an amino acid sequence of butyrylcholinesterase selected for the focused library of butyrylcholinesterase variants provided by the invention are shown in ~~Table 1~~ **Table 2**.

¶ 67:

Methods for preparing libraries containing diverse populations of various types of molecules such as peptides, peptoids and peptidomimetics are well known in the art (see, for example, Ecker and Crooke, ~~*Biotechnology*~~ ***Biotechnology*** 13:351-360 (1995), and Blondelle et al., *Trends Anal. Chem.* 14:83-92 (1995), and the references cited therein, each of which is incorporated herein by reference; see, also, Goodman and Ro, *Peptidomimetics for Drug Design*, in "Burger's Medicinal Chemistry and Drug Discovery" Vol. 1 (ed. M.E. Wolff; John Wiley & Sons 1995), pages 803-861, and Gordon et al., *J. Med. Chem.* 37:1385-1401 (1994), each of which is incorporated herein by reference). Where a molecule is a peptide, protein or fragment thereof, the molecule can be produced *in vitro* directly or can be expressed from a nucleic acid, which can be produced *in vitro*. Methods of synthetic peptide chemistry are well known in the art.

¶ 70:

The generation of a library of nucleic acids encoding butyrylcholinesterase variants can be by any means desired by the user. Those skilled in the art will know what methods can be used to generate libraries of nucleic acids encoding butyrylcholinesterase variants. For example, butyrylcholinesterase variants can be generated by mutagenesis of nucleic acids encoding butyrylcholinesterase using methods well known to those skilled in the art (*Molecular Cloning: A Laboratory Manual*, Sambrook et al., eds., Cold Spring Harbor Press, Plainview, NY (1989)). A library of nucleic acids encoding butyrylcholinesterase variants of the invention can be randomized to be sufficiently diverse to contain nucleic acids encoding every possible naturally occurring amino acid at each amino acid position of butyrylcholinesterase. Alternatively, a library of nucleic acids can be prepared such that it contains nucleic acids encoding every possible naturally ~~occuring~~ **occurring** amino acid at each amino acid only at positions located within a region of butyrylcholinesterase predicted or determined to be important for cocaine hydrolysis activity.

¶ 85:

Following the expression of a library of butyrylcholinesterase variants in a mammalian cell line, randomly selected clones can be sequenced and screened for increased cocaine hydrolysis activity. Methods for sequencing selected clones are well known to those of skill in the art and are described, for example, in Sambrook et al., *supra*, 1992, and in ~~Ansubel~~ Ausubel et al., *supra*, 1998. Selecting a suitable method for measuring the cocaine hydrolysis activity of a butyrylcholinesterase variant depends on a variety of factors such as, for example, the amount of the butyrylcholinesterase variant that is available. The cocaine hydrolysis activity of a butyrylcholinesterase variant can be measured, ~~for example,by~~ for example, by spectrophotometry, by a microtiter-based assay utilizing a polyclonal anti-butyrylcholinesterase antibody to uniformly capture the butyrylcholinesterase variants and by high-performance liquid chromatography (HPLC).

¶ 87:

Clones expressing a butyrylcholinesterase variant with increased cocaine hydrolysis activity can be used to ~~established~~ establish larger-scale cultures suitable for purifying larger quantities of the butyrylcholinesterase. A butyrylcholinesterase variant of interest can be cloned into an expression vector and used to transfect a cell line, which can subsequently be expanded. Those skilled in the art will know what type of expression vector is suitable for a particular application. A butyrylcholinesterase variant exhibiting increased cocaine hydrolysis activity can be cloned, for example, into an expression vector carrying a gene that confers resistance to a particular chemical agent to allow positive selection of the transfected cells. An expression vector suitable for transfection of, for example, mammalian cell lines can contain a promoter such as the cytomegalovirus (CMV) promoter for selection in mammalian cells. As described herein, a butyrylcholinesterase variant can be cloned into a mammalian expression vector and transfected into Chinese Hamster Ovary cells (CHO). Expression vectors suitable for expressing a butyrylcholinesterase variant are well known in the art and commercially available.

¶ 93:

As ~~desciбed~~ **described** herein, a butyrylcholinesterase variant exhibiting increased cocaine hydrolysis activity can hydrolyze a cocaine-based butyrylcholinesterase substrate *in vitro* as well as *in vivo*. A cocaine-based butyrylcholinesterase substrate can be contacted with a butyrylcholinesterase variant of the invention *in vitro*, for example, by adding the substrate to supernatant isolated from cultures of butyrylcholinesterase variant library clones. Alternatively, the butyrylcholinesterase variant can be purified prior to being contacted by the substrate. Appropriate medium conditions in which to contact a cocaine-based substrate with a butyrylcholinesterase variant of the invention are readily determined by those skilled in the art. For example, 100 μM cocaine in 10mM Tris at pH 7.4 can be contacted with a butyrylcholinesterase variant at 37° C. As described below, butyrylcholinesterase variants from culture supernatants can further be immobilized using a capture agent, such as an antibody prior to being contacted with a substrate, which allows for removal of culture supernatant components and enables contacting of the immobilized variants with substrate in the absence of contaminants. Following contacting of a butyrylcholinesterase variant of the invention with a cocaine-based substrate, cocaine hydrolysis activity can be measured by a variety of methods known in the art and described herein, for example, by high-performance liquid chromatography or the isotope tracer cocaine hydrolysis assay.

¶ 96:

A butyrylcholinesterase variant can be delivered systemically, such as intravenously or intraarterially. A butyrylcholinesterase variant can be provided in the form of isolated and substantially purified ~~polypetides~~ **polypeptides** and polypeptide fragments in pharmaceutically acceptable formulations using formulation methods known to those of ordinary skill in the art. These formulations can be administered by standard routes, including for example, topical, transdermal, intraperitoneal, intracranial, intracerebroventricular, intracerebral, intravaginal, intrauterine, oral, rectal or parenteral (e.g.,

intravenous, intraspinal, subcutaneous or intramuscular) routes. In addition, a butyrylcholinesterase variant can be incorporated into biodegradable polymers allowing for sustained release of the compound useful for treating ~~individual~~ **individuals** symptomatic of cocaine addiction. Biodegradable polymers and their use are described, for example, in detail in Brem et al., *J. ~~Neurosurg.~~ Neurosurg.* 74:441-446 (1991), which is incorporated herein by reference.

¶ 104:

This example describes the discovery and characterization of the butyrylcholinesterase variant designated SEQ ID NO: 2, in which Alanine (A) at amino acid position 328 of human butyrylcholinesterase is replaced with ~~Tryptophane~~ **Tryptophan** (W). The A328W butyrylcholinesterase variant designated SEQ ID NO: 2 exhibits a 15-fold increase in cocaine hydrolysis activity compared to human butyrylcholinesterase.

¶ 107:

The structures of (-)-cocaine and (+)-cocaine were retrieved from the Cambridge Structural Database where its code-names are COCAIN10 and COCHCL. The HCl molecule was deleted from COCHCL so that all computations were done with the base form of cocaine. Before the FlexiDock program was run, cocaine was manually aligned with butyrylcholine in the model of human butyrylcholinesterase as described by Harel et al., *Proc. Natl. Acad. Sci. USA*, 89: 10827-10831 (1992). Manual alignment was performed so that the tropane ring of cocaine faced the ~~Tryptophane~~ **Tryptophan** residue (W) at amino acid position 82 of butyrylcholinesterase, the carboxyl group of the benzoic ester of cocaine was within 1.5Å of the Serine (S) residue at amino acid position 198 of butyrylcholinesterase, and the benzene ring of cocaine was in the acyl binding pocket of butyrylcholinesterase. In the FlexiDock the binding pocket was defined as all amino acids within 4Å of butyrylcholine. After defining the binding pocket, the butyrylcholine molecule was extracted. All atoms in the binding pocket, except atoms in rings and double bonded

atoms, were defined as rotatable, thus yielding 124 rotatable bonds in butyrylcholinesterase and 7 rotatable bonds in cocaine.

¶ 111:

The 1.8-kb fragments constituting the butyrylcholinesterase variants were cloned into the plasmid pGS and resequenced to make sure the desired mutation was present. The plasmid pGS is identical with pRc/CMV (Invitrogen, Carlsbad, CA) except that the Neo gene has been replaced by rat glutamine ~~synthesase~~ **synthetase**.

¶ 115:

To purify the butyrylcholinesterase variants, the culture medium corresponding to each variant was filtered through Whatman #1 filter paper (Whatman Inc., Clifton, NJ) on a Buchner funnel. The filtrate was poured through a chromatography column (XK50/30, Pharmacia Biotech, ~~Piscataway~~ **Piscataway**, NJ) packed with 100ml of affinity gel procainamide-Sepharose 4B. The butyrylcholinesterase variants stick to the affinity gel during loading so that 20mg of enzyme that was previously in 20 liters was concentrated in 100ml of affinity gel. The affinity gel was subsequently washed with .3M sodium chloride in 20mM potassium phosphate pH 7.0 and 1mM EDTA to elute contaminating proteins. Next, the affinity gel was washed with buffer containing 20mM potassium phosphate and 1 mM EDTA pH 7.0 to reduce the ionic strength. Finally, the ~~butyrylcholinesterase~~ **butyrylcholinesterase** ~~variants was~~ **variants were** eluted with 250ml of 0.2M procainamide in buffer.

¶ 116:

To further purify the butyrylcholinesterase variants and remove the procainamide a second purification step was performed. The butyrylcholinesterase variants recovered in the first purification step were diluted 10-fold with buffer (20 mM TrisCl, 1 mM EDTA pH 7.4)

to reduce the ionic strength to about 0.02M. The diluted enzyme was loaded onto a column containing 400ml of the weak anion exchanger DE52 (Whatman, Clifton, NJ). At this low ionic strength the butyrylcholinesterase variant sticks to the ion exchange gel. After loading was complete the column was washed with 2 liters of buffer containing 20mM TrisCl and 1mM EDTA pH 7.4 until the absorbency of the eluant at 280nm was nearly zero, indicating that the procainamide had washed off. Subsequently, the butyrylcholinesterase variants were eluted from the column with a salt gradient from 0 to 0.2M NaCl in 20mM TrisCl pH 7.4. Following the elution of the butyrylcholinesterase variants 10ml fractions were collected for each variant using a fraction collector. Activity assays were performed to identify the peak containing butyrylcholinesterase variant. SDS gel electrophoresis was performed to determine the purity of each butyrylcholinesterase ~~variants~~ **variant**, which was determined to be approximately 90%.

¶ 123:

At the conclusion of the isotope tracer assay, an aliquot of the reaction mix is acidified in order to take advantage of the solubility difference between the product and the substrate at pH 3.0. At pH 3.0, [3H]-benzoic acid (pKa=4.2) is soluble in a scintillation cocktail consisting of 2,5-diphenyloxazole (PPO) and ~~[1,4-bis-2-(4-methyl-5-phenyloxazolyl0-benzene]~~ **[1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene]** (POPOP) (PPO-dimethyl-POPOP scintillation fluor, Research Products International Corp., Mt. Prospect, IL) while [3H]-cocaine is not. The signal generated by acidified reaction mixture from enzyme blanks was less than 2% of the total dpm ~~paleed~~ **placed** in the fluor, consistent with cocaine being insoluble in PPO-dimethyl-POPOP.

¶ 137:

The seven regions of butyrylcholinesterase selected for focused library synthesis (summarized in Table 2) span residues that include the 8 aromatic active site gorge residues (W82, W112, Y128, W231, F329, Y332, W430 and Y440) as well as two of the catalytic

triad residues. The integrity of intrachain disulfide bonds, located between ~~^{65}Cys-^{92}Cys, ^{252}Cys-^{263}Cys, and ^{400}Cys-^{519}Cys~~ ^{65}Cys-^{92}Cys, ^{252}Cys-^{263}Cys, and ^{400}Cys-^{519}Cys is maintained to ensure functional butyrylcholinesterase structure. In addition, putative glycosylation sites (N-X-S/T) located at residues 17, 57, 106, 241, 256, 341, 455, 481, 485, and 486 also are avoided in the library syntheses. In total, the seven focused libraries span 79 residues, representing approximately 14% of the butyrylcholinesterase linear sequence, and result in the expression of about 1500 distinct butyrylcholinesterase variants.

¶ 138:

Libraries of nucleic acids corresponding to the seven regions of human butyrylcholinesterase to be mutated are synthesized by codon-based mutagenesis, as described above and as depicted schematically in Figure 7. Briefly, multiple DNA synthesis columns are used for synthesizing the oligonucleotides by ß-cyanoethyl phosphoramidite chemistry, as described previously by Glaser et al., *supra*, 1992. In the first step, trinucleotides encoding for the amino acids of butyrylcholinesterase are synthesized on one column while a second column is used to synthesize the trinucleotide NN(G/T), where N is a mixture of dA, dG, dC, and dT cyanoethyl ~~phosphoramadites~~ phosphoramidites. Using the trinucleotide NN(G/T) results in thorough mutagenesis with minimal degeneracy, accomplished through the systematic expression of all twenty amino acids at every position.

¶ 139:

Following the synthesis of the first codon, resins from the two columns ~~are be~~ are mixed together, divided, and replaced in four columns. By adding additional synthesis columns for each codon and mixing the column resins in the manner illustrated in Figure 7, pools of degenerate oligonucleotides will be segregated based on the extent of mutagenesis. The resin mixing aspect of codon-based mutagenesis makes the process rapid and cost-effective because it eliminates the need to synthesize multiple oligonucleotides. In the

present study, the pool of oligonucleotides encoding single amino acid mutations ~~are~~ **is** used to synthesize focused butyrylcholinesterase libraries.

¶ 140:

The oligonucleotides encoding the butyrylcholinesterase variants containing a single amino acid mutation is cloned into the double*lox* targeting vector using oligonucleotide-directed mutagenesis (Kunkel, *supra*, 1985). To improve the mutagenesis efficiency and diminish the number of clones expressing wild-type butyrylcholinesterase, the libraries are synthesized in a two-step process. In the first step, the butyrylcholinesterase DNA sequence corresponding to each library site is deleted by hybridization mutagenesis. In the second step, uracil-containing single-stranded DNA for each deletion mutant, one deletion mutant corresponding to each library, is isolated and used as template for synthesis of the libraries by oligonucleotide-directed mutagenesis. This approach has been used routinely for the synthesis of antibody libraries and results in more uniform mutagenesis by removing annealing biases that potentially arise from the differing DNA sequence of the mutagenic oligonucleotides. In addition, the two-step process decreases the frequency of wild-type sequences relative to the variants in the libraries, and consequently makes library screening more efficient by eliminating repetitious screening of clones encoding wild-type ~~butyrylcholinest~~ **butyrylcholinesterase.**

¶ 141:

The quality of the libraries and the efficiency of mutagenesis is characterized by obtaining DNA sequence from approximately 20 randomly selected clones from each library. The DNA sequences demonstrate that mutagenesis ~~occurrs~~ **occurs** at multiple positions within each library and that multiple amino acids were expressed at each position. Furthermore, DNA sequence of randomly selected clones demonstrates that the libraries contain diverse clones and are not dominated by a few clones.

¶ 152:

As described previously, the sequencing demonstrates uniform introduction of the library and the diversity of mammalian transformants resembles the diversity of the library in the doublelox targeting vector following transformation of bacteria. ~~Table 6. Identification and characterization of butyrylcholinesterase variants, with enhanced cocaine hydrolase activity.~~

Table 6. Identification and characterization of butyrylcholinesterase variants, with enhanced cocaine hydrolase activity.

¶ 169:

Six groups of six rats each are used in this study. The rats are Sprague-Dawley males, weighing 250-275g upon receipt in the vivarium, which is maintained on a 12 hour light-dark cycle. The rats have food and water available *ad libitum* at all times. Prior to treatment the rats are fitted with femoral arterial and venous catheters and permitted to recover. Subsequently, the rats are treated with varying amounts of the butyrylcholinesterase variants (0.35, 1.76, or 11.8 mg/kg) or equivalent volumes of saline 15 minutes prior to the co-infusion of catecholamines and cocaine (1 mg/kg/min). The infusion is for 16 minutes to deliver the LD_{90} of cocaine, unless the animals expire sooner. Based on the relative catalytic efficiencies of wild-type butyrylcholinesterase and the previously described catalytic antibody (Mets et al., *supra*, 1998), it is anticipated that increasing doses of butyrylcholinesterase confer increased survival rate to the rats relative to the saline controls and that the highest butyrylcholinesterase dose (11.8 mg/kg) protects all the animals. A butyrylcholinesterase variant that hydrolyzes cocaine 10-fold more efficiently *in vitro* ~~is be expected~~ **is expected** to confer protection to all of the animals at a lower dose (1 mg/kg, for example).

¶ 179:

Drug discrimination is relevant to the subjective effect of cocaine in clinical situations and antagonism of cocaine discrimination following pretreatment is considered clear evidence of therapeutic potential (Holtzman, ~~*Modern*~~ ***Modern*** *Methods in Pharmacology, Testing and Evaluation of Drug Abuse*, Wiley-Liss Inc., New York, (1990); Spealman, *NIDA Res. Mon.* 119: 175-179 (1992)). The most frequently used procedure to establish and evaluate the discriminative stimulus effect of drugs is to train animals in a controlled operant procedure to use the injected drug as a stimulus to control distribution of responding on two levers. Dose- effect curves consisting of distribution of the responses on the "drug-associated" lever as a function of drug dose are easily generated. These cocaine dose-effect curves can be altered by the administration of a competitive antagonist. The amount of the shift of the curve and time required for the original sensitivity of the animal to cocaine to return are useful data for evaluating the potential therapeutic use of wild-type butyrylcholinesterase and the optimized variant. The discriminative stimulus effects of cocaine in rat models have been used to evaluate the therapeutic potential of dopamine reuptake inhibitors, as well as agonists and antagonists to the dopamine receptors (Witkin et al., *J. Pharmacol. Exp. Ther.* 257: 706-713 (1989); Kantak et al., *J. Pharmacol. Exp. Ther.* 274: 657-665 (1995); Barret and Appel, *Psychopharmacology* 99: 13-16 (1989); Callahan et al., *Psychopharmacology* 103: 50-55 (1991)).